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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This project attempts to probe interrelationships between the central nervous system, immune and endocrine systems. The key departure was the discovery that certain synthetic copolymers stimulate hyperplasia of cortical lymphocytes in the thymus and increased motor behavior. These copolymers, polyphores, are monovalent cation-selective ionophores. During the past year, we developed evidence that T150R1 represents a new class of immunoendocrine modulators. It appears to induce corticosteroids by both a pituitary and an extra-pituitary mechanisms. It may bypass the pituitary either by acting directly on the adrenals, or by acting indirectly on immune cells, such as lymphocytes or macrophages, to induce ACTH or interferon, that can stimulate the adrenals. Its mechanism of action may be by altering cation flux in cells within the hythalamo-pituitary-adrenal axis, as well as cells of the immune system. Many studies attempting to relate behavioral and immunologic changes induced by the copolymers were conducted. Several interesting observations were made, but the system proved too complex for definitive analysis. The goals for next year are:

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Block 19 Abstract, continued

1. We have spent a great deal of time and effort on studies relating behavior to thymic size as a followup to our initial observations in this area. We have worked through enough variables to be convinced that the phenomenon is real but the variability in the system is so great as to preclude decisive dissection. Consequently, during the final year of this project, we will concentrate on leads suggested by earlier portions of this study which are likely to provide definitive answers to important questions. In particular, we will investigate the relationship between specific brain structures known to be involved in mood and motivation and the immune system. We will conduct more ICSS investigations, examining non-hypothalamic sites. We will also continue some preliminary work on brain catacholamine systems and the immune system.

2. Complete studies of the effects of copolymer on pituitary and extra-pituitary ACTH secretion. Studies in vivo will involve dexamethasone suppression, an effort to evaluate the ability of the copolymer to induce extra-pituitary ACTH. In the cell culture model, studies with the copolymers will be carried out in parallel with phorbol esters and various metabolic inhibitors in an effort to more clearly define the mechanisms involved. The model for these studies will be investigations on HL60 cells which were recently completed by a student in this department for the Ph.D. thesis.

3. Investigation of hematopoietic effects of the copolymers. Splenomegaly has been consistently observed in animals injected with the copolymers. Histologic sections have suggested that this is primarily hematopoietic with both red and white blood cells present. We will characterize the time course and nature of this effect on the hematopoietic system. This work will be done in collaboration with Dr. Elliot Winton who has established procedures for assaying various types of stem cells in culture.

Annual Report

# Investigations of the biologic activity and structure function relationships of synthetic polymers which act as hormones, behavior modifiers and thymic immunomodulators.

**O.N.R. Contract #N00014-86-K-0456**

## Introduction

In the course of investigating synthetic immunomodulating agents, we found that a particular alkoxylated amine copolymer, 'Polyphore 32:5', produced a rapid and sustained increase in the size of the thymus in rats and mice. This increase was due to cortical T lymphocytes. T lymphoid cell numbers in peripheral lymphoid tissues were also increased. Injection of polyphore produced a number of other effects including increased motor activity and diuresis which suggested that they affected the central nervous system in addition to the thymus. Studies in vitro demonstrated that polyphores were ionophores with selectivity for monovalent cations and that they were potent stimulators of histamine release from mast cells. Finally, certain structural and physicochemical properties suggested to us that polyphores might be analogues of substance P or somatostatin. Molecules which affect sodium gradients across cell membranes and mimic the activity of neuropeptides might be expected to have diverse effects on excitable cell systems. This project was designed to characterize the effects of polyphore copolymers on the thymus and other elements of the immune system and to evaluate selected behavioral effects.

## Chemical structure and nomenclature of polyphore copolymers:

The compounds used in these studies are nonionic block copolymer surfactants which consist of a central initiator of ethylenediamine linked to blocks of hydrophilic polyoxyethylene (POE) flanked by hydrophobic blocks of polyoxypropylene (POP) (Figure 1). They comprise a series of chemically synthesized copolymers which vary in length, relative proportions of POP and POE and molecular configuration. The copolymers, designated polyphores, are manufactured by BASF as Reverse Tetronic Polyols or reverse octoblock copolymers. Some are also available from

CytRx Corporation, Atlanta, Georgia. The studies in this project have concentrated on Reverse Tetronic Polyol T150R1 which is also known as Polyphore 32:5. Each of the four chains of POE have an average of five ethylene oxide moieties which each of the POP chain have an average of 32 moieties.

**Structures and Commercial Names of the Copolymers**

| BASF name | CytRX name | M.W. | Structure |
|---|---|---|---|
| T110R1 | Polyphore 21:3 | 5520 | 21 3 X 3 21<br>21 3 X 3 21 |
| T130R1 | Polyphore 27:4 | 6800 | 27 4 X 4 27<br>27 4 X 4 27 |
| T130R2 | Polyohore 27:10 | 7740 | 27 10 X 10 27<br>27 10 X 10 27 |
| T150R1 | Polyphore 32:5 | 8000 | 32 5 X 5 32<br>32 5 X 5 32 |
| T150R4 | Polyphore 32:26 | 11800 | 32 26 X 26 32<br>32 26 X 26 32 |
| Tetronic T1501 | | 8000 | 5 32 X 32 5<br>5 32 X 32 5 |

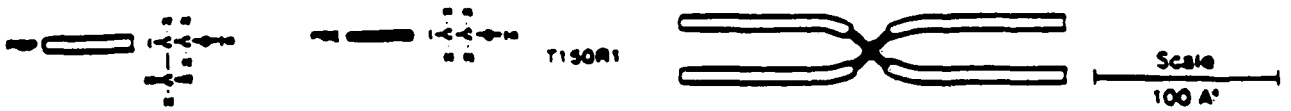



**Figure 1:** The copolymers are composed of blocks of polyoxyethylene (POE) and polyoxypropylene (POP) attached to a central ethylene-diamine group. The numbers in the structure column represent the average number of groups of oxyethylene and oxypropylene (underlined) in each block rounded to the nearest integer. The diagram is drawn to scale showing the four chain structure of the copolymers.

## Ionophore activity

The activity of the polyphores as ionophores has been demonstrated in several systems. First, using artificial lipid membranes, we demonstrated that the polyphores were able to transport sodium, potassium and hydrogen across an artificial lipid membrane in a voltage dependent fashion wit kinetics which suggested a carrier rather than a channel-forming mechanism. Secondly, using red blood cells and radioactive cation isotopes, we demonstrated that the polyphores could

mediate the exchange of external sodium with potassium inside red blood cells. In this system, the polyphores had approximately equal affinity for sodium and potassium, but no demonstrable affinity for calcium.

Extensive studies were done evaluating the ability of polyphores to produce histamine release from murine peritoneal mast cells and human peripheral blood basophils. The polyphores produces an energy dependent active release of histamine from both types of cells. The polyphores acted synergistically with anti IgE, concanavalin A and phorbol esters, but not with the calcium ionophore A23187. Even though the polyphores had no demonstrable affinity for calcium, calcium in the medium was required for release of histamine. Each of the hydrophobic copolymers tested had qualitatively similar activities as ionophores, but they differed in intensity. Polyphore 27:10 (T130R2) was the most effective ionophore. Polyphore 32:5 (T150R1) is much less toxic and in preliminary studies demonstrated marked stimulatory effects on cortical thymocytes in vivo. We proposed that the ionophores caused an increase of intracellular sodium which was exchanged for calcium and that an increased level of intracellular calcium was responsible for the increased excitability of these cells. This mechanism has potential activity in many types of cells.

## Specific Aims for Year 1

1. Optimize the dose and time parameters for production of thymic hyperplasia and behavioral changes by Polyphore 32:5 in Lewis rats.

2. Immunochemically and functionally characterize the effects of Polyphore 32:5 on thymus and peripheral lymphoid tissue.

3. Investigate hormonal mechanisms which might mediate the effects of Polyphore 32:5.

## Summary of Progress for Year 1

A number of variables including the species and strain of animal, dose and route of administration of polyphore were evaluated for their effects on immunological and behavioral measurements. It became evident that additional variables related to stress confounded the interpretation of the results. These included the location of the cages (temperature, light), food and/or water deprivation, single vs. group housing, footpad inflammation, co-existing infections, etc. We observed that the most positive effects of polyphores had been observed in animals who had

undergone severe stress. The positive results could not be attributed to random variation or noise in the system. When present, they were quite clear and distinct from controls. Parallel experiments with adrenalectomized and hypophysectomized mice suggested that the effects required intact adrenal glands, but not the hypophysis. Consequently, we have formed a modified hypothesis to be evaluated during the coming year; namely that the polyphores act to modulate the effects of stress.

## Specific Aims for Year 2

Animals will be treated with stressful conditions in a controlled fashion in an effort to test the hypothesis that the copolymer modifies the stress reaction. Several stressful stimuli will be evaluated. Assays during the coming year will include hormonal measurements in addition to the behavioral, gross and microscopic pathologic studies similar to those done this year. Once we have identified the conditions to produce the changes in the immune system and behavior, other studies as outlined in the application will be carried out to evaluate them further. In parallel experiments we will evaluate the effects of the copolymers on pituitary and adrenal functions in adrenalectomized, hypophysectomized and normal rats.

## Progress Year 2

### Immunoendocrine effects of synthetic block copolymer T150R1

T150R1 is an 8000 dalton copolymer ionophore. When injected in mice as an oil-in-water emulsion, 2.5 mg T150R1 caused a profound thymic involution for 2-7 days. This was followed by normalization then hyperplasia. T150R1 also caused splenomegaly with stimulation of the red pulp as well as a long-lasting peripheral blood neutrophilia.

To test whether T150R1 exhibited endocrine effects through the pituitary-adrenal axis, adrenalectomized or hypophysectomized mice were injected with T150R1. Thymus size, spleen size, and serum corticosterone level were measured. Groups of female or male BALB/c mice 6-8 weeks of age were used. In studies where adrenalectomized or hypophysectomized mice were used, surgery had been performed by the animal supplier. Adrenalectomized mice were maintained with saline water. Mice were injected in the footpads with 2.5 mg copolymer T150R1, in an oil-in-water emulsion consisting of Drakeol and PBS with 0.5 mg/mL of Bovine Serum Albumin. Control groups were non-injected normal mice, and in some cases vehicle injected mice (emulsion excluding T150R1).

Mice were sacrificed by $CO_2$ asphyxiation. At autopsy, thymuses and spleens were carefully freed from adjacent tissues then accurately weighed. These organs were processed for paraffin embedding, sectioning, and Hematoxylin & Eosin staining. Commercial kits to measure corticosterone were used. Mouse sera were collected from retroorbital plexus. Attention was given not to agitate the animals prior to bleeding.

The immune effects of T150R1 on the humoral system were evaluated by immunizing copolymer treated mice with T-dependent and T-independent antigens at two time points: day 2 post-T150R1, when the thymus is involuted, and day 21 post-T150R1, when thymus hyperplasia is at its peak. Serum IgG or IgM antibody titers were measured.

To test the humoral response to T-dependent antigens, 100 µg TNP-Ficoll were injected intraperitoneally at similar time points. Anti-TNP antibodies were measured by an ELISA assay.

Induction of ACTH secretion in vitro: A murine pituitary tumor cell line, AtT20, from the American Type Culture Collection was used to investigate the ability of copolymer T150R1 to induce the secretion of ACTH in vitro. Cell cultures were split placing 100,000 cells in each well of a microtiter tray in tissue culture medium. After 24 hours acclimation, copolymer T150R1 or T130R2 were added for one hour. The culture supernatant was then assayed for ACTH by a radioimmunoassay. The results were as follows:

**Table 1**

| | |
|---|---|
| Cells alone | 5000 ng/mL |
| Cells + 20 µg T130R2 | 10500 ng/mL |
| Cells + 20 µg T150R1 | 10000 ng/mL |
| Cells + $10^{-7}$M TPA | 6000 ng/mL |

The two copolymers significantly increased secretion of ACTH by pituitary cells in culture.

Key Findings

1. The rapid thymic involution, seen early after T150R1 administration, was found to be corticosteroid mediated. Subtotal adrenalectomy proportionally diminishes this effect (Figure 2).

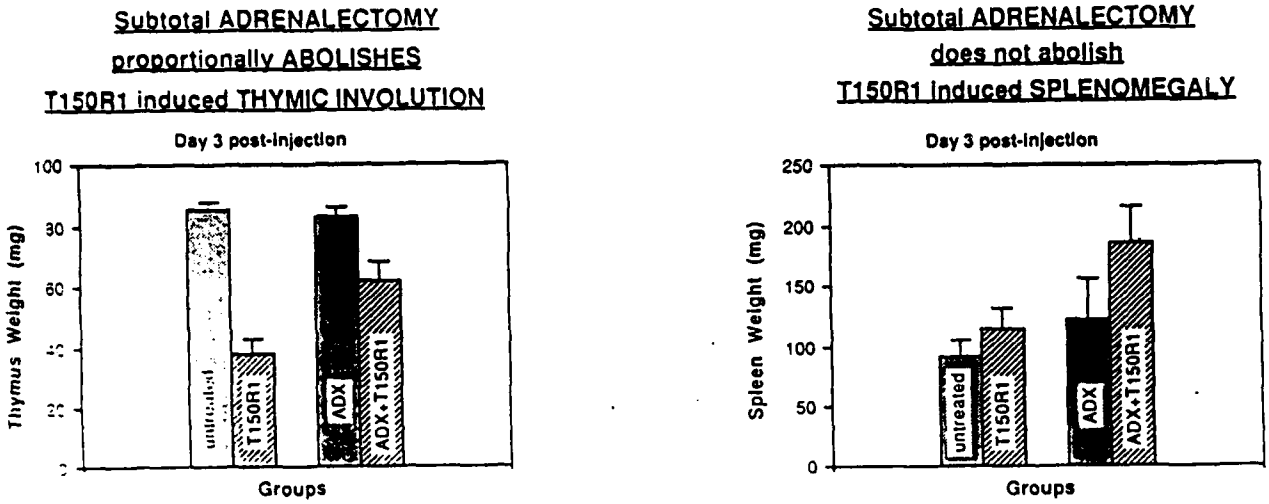



**Figure 2:** Groups of 3 BALB/c female mice, normal or adrenalectomized (ADX) received 2.5 mg T150R1 oil-in water emulsion in the footpads. They were compared to non-injected normal or ADX mice. Thymus and spleen weights were determined at day 3 and are expressed as X + SD. In all comparisons T150R1 treated mice were significantly different from untreated. Histologic examination showed adrenal remnants in some animals, hence subtotal adrenalectomy.

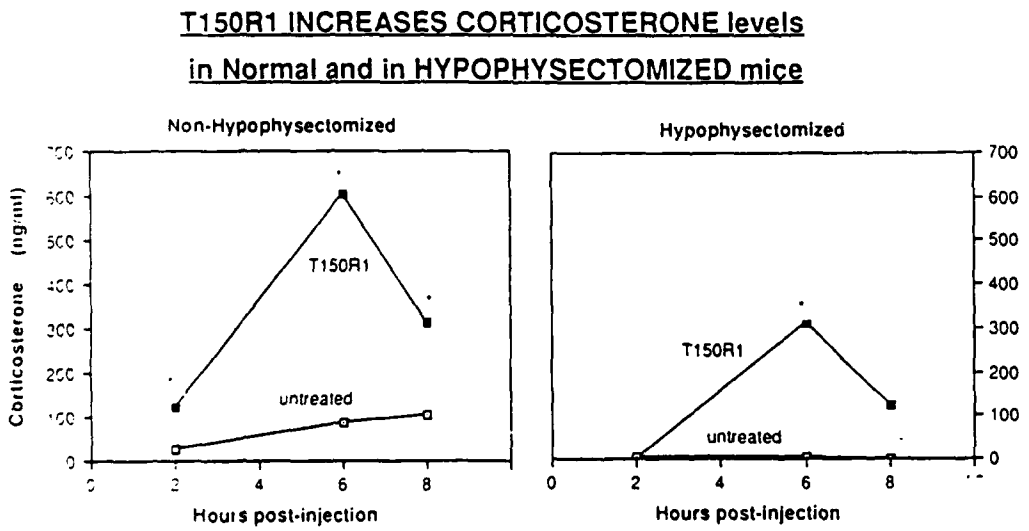
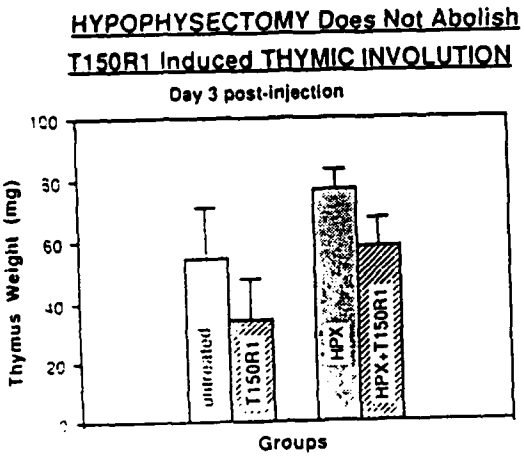



**Figure 3:** Groups of 3 BALB/c males, normal and hypophysectomized (HPX) were injected with 2.5 mg T150R1 oil-in water emulsion in the footpads. They were compared to non-injected normal or HPX mice. Corticosterone levels were determined by RIA, the asterisks indicates statistically significant differences. T150R1-induced changes in corticosterone levels peaked at 6 hours. Hypophysectomy did not abolish the increase suggesting that T150R1 bypasses the pituitary.



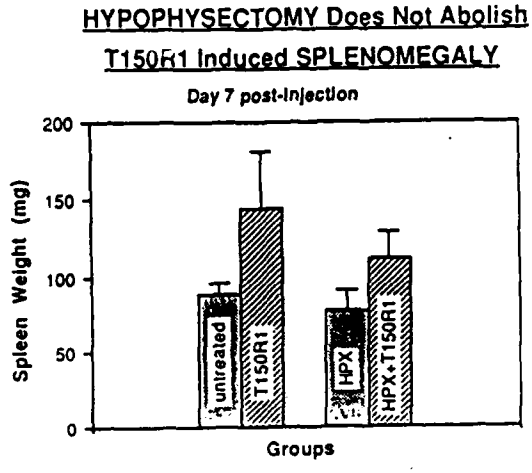
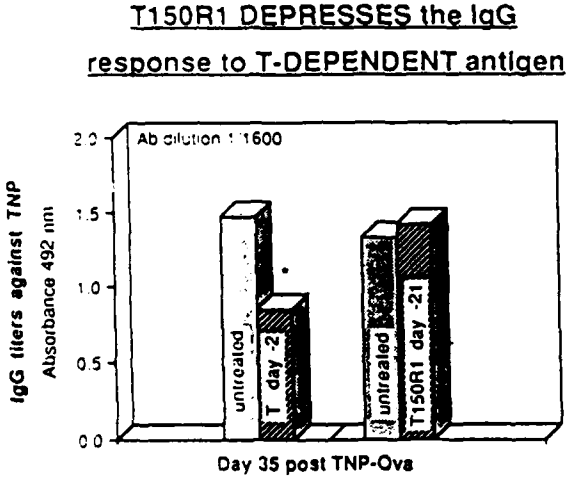




**Figure 4:** Groups of 3 BALB/c mice, normal or hypophysectomized (HPX) received 2.5 mg T150R1 oil-in water emulsion in the footpads. They were compared to non-injected normal or ADX mice. Thymus weights were determined at day 3 and spleen weights at day 7. They are expressed as X+SD. In all comparisons T150R1 treated mice were significantly different from untreated. Complete hypophysectomy was verified by microscopic examination.



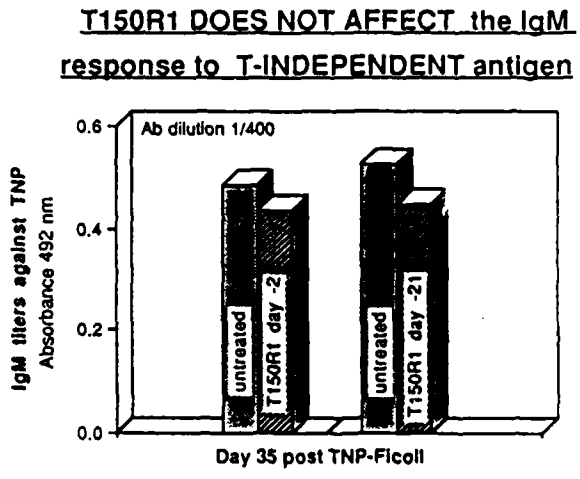




**Figure 5:** Groups of 5 BALB/c mice were injected with 2.5 mg T150R1 oil-in water emulsion in the footpads. Two days or 21 days later, they were immunized IP either with 100 µg TNP-ovalbumin in 0.1 mL CFA, or with 18 µg TNP-Ficoll. An ELISA assay was used to measure anti-TNP antibodies. The asterisk indicates a statistically significant difference.

2. T150R1 increases serum corticosterone levels via dual pituitary and extra-pituitary mechanisms. Hypophysectomy diminishes, but does not abolish thymic involution (Figure 3).

3. Serum corticosterone levels increase by 2 hours and peak at 6 hours in non-hypophysectomized T150R1 injected mice. The early (2 hours) increase is pituitary mediated and is abolished by hypophysectomy. The late increase is still seen in hypophysectomized T150R1 injected mice, indicating that the late (6 hours) increase in corticosterone levels is not due to pituitary involvement (Figure 4).

4. The humoral response to T-dependent antigens is depressed, but the response to T-independent antigens is not affected in copolymer treated mice. This is compatible with results which would be predicted from the increased serum corticosteroids in these mice (Figure 5).

5. The copolymers can directly induce the secretion of ACTH by pituitary cells (Table 1).

Summary

T150R1 represents a new class of immunoendocrine modulators. It appears to induce corticosteroids by both a pituitary and an extra-pituitary mechanisms. It may bypass the pituitary either by acting directly on the adrenals, or by acting indirectly on immune cells, such as lymphocytes or macrophages, to induce ACTH or interferon, that can stimulate the adrenals. Its mechanism of action may be by altering cation flux in cells within the hythalamo-pituitary-adrenal axis, as well as cells of the immune system.

## Behavioral Studies

Behavioral Studies in Nonstressed Rats

In the first year of this project a number of variables including species and strain of animal and route of administration of polyphore were evaluated for their effects on immunological and behavioral measurements. A number of variables were examined. These included: location of the cages, food and/or water deprivation, single or group housing, footpad or subcutaneous neck injection site, and the stress of co-existing infections. In general the experiments carried out with healthy animals housed under optimum conditions failed to show significant effects on behavior, organ weights, blood chemistries or other parameters we measured. However, there seemed to be a tendency for sick animals housed under suboptimal conditions to show significant effects. In the

past year examination of some of the previous variables was expanded and new variables were considered. The variables included animal species, strain, and age, copolymer dose, and food and water deprivation. It was found that, although definite trends were noted, there were no statistical differences between copolymer-treated and control groups.

Experiments:

In all of the past year's experiments, animals were acclimatized to the animal colony for at least one week prior to the assessment of baseline measurements. All rats were albino male, singly-housed and approximately 3 1/2 months old when injections were administered. Strain is mentioned with each experiment below. In all cases the vehicle was olive oil and injections were administered subcutaneously in the dorsum of the neck. Experimental protocols were similar in this series of experiments, so the results from them were pooled for several of the analyses.

The first two experiments were designed to assess the effects of differing dosages of Polyphore 32:5 on rats and mice. In the first experiment 13 Lewis rats were given injections of 0, 5, or 10 mg of the copolymer. Baseline measurements of spontaneous activity, body weight and water intake were taken for two weeks prior to the injections, and the same measurements were taken for four weeks after injections. Animals were killed 29 days after injections and hearts, spleens and thymuses were weighed. No drug dosage group differed significantly from its baseline control or the vehicle control groups on any of the variables. However, 5 mg copolymer came closest to producing an increase in water intake and activity when compared to the other dosages in this experiment and the 2.5 mg doses used in last year's experiments. In the second experiment 34 group-housed, female ICR mice were injected with 0, 2.5, 5 or 10 mg Polyphore. Water intake and waste material (as ascertained by weighing the litter) were measured until the mice were killed 31 days after the injection date. Although no significant differences were noted between the groups, the 5 mg dose showed the greatest effect, appearing to increase urine output and spleen size. It was concluded that 5 mg Polyphore would give us the largest effects.

At this point we noted that we were getting strong and consistent trends (larger animals; larger spleens, thymuses and hearts; increased spontaneous activity, water intake and urine outputs in the drug groups), but the variability between the rats was so high that statistical significance was not obtained. Use of the inbred Lewis strain of rats did not attenuate the inter-rat variability. Consequently, it was decided to

use Sprague-Dawley rats in the rest of the experiments since this strain seemed to show the greatest effects.

The next three experiments were designed primarily to increase the number of animals in the experiments and obtain further behavioral measurements. In these experiments 46 Sprague-Dawley rats were used and baseline measurements of activity, body weight and water were taken for two weeks prior to injections. The animals were killed for tissue samples at five weeks post-injection. The copolymer groups drank less than the vehicle control groups in one experiment, more in another and the same in the third. Spontaneous activity results from these three experiments and the 5 mg copolymer rat group of the earlier dosage experiment were pooled. Vehicle control rats showed a decrease in activity when compared to their last baseline week. Polyphore-treated rats showed some increase or less decrease than seen in controls when compared to their baseline week (Figure 6). The difference between these two groups was most marked in the first week after injections. However, neither the repeated-measure analysis of variance across the five weeks nor any individual week comparisons were significant.

**Effects of Polyphore 32:5 on spontaneous activity in rats**

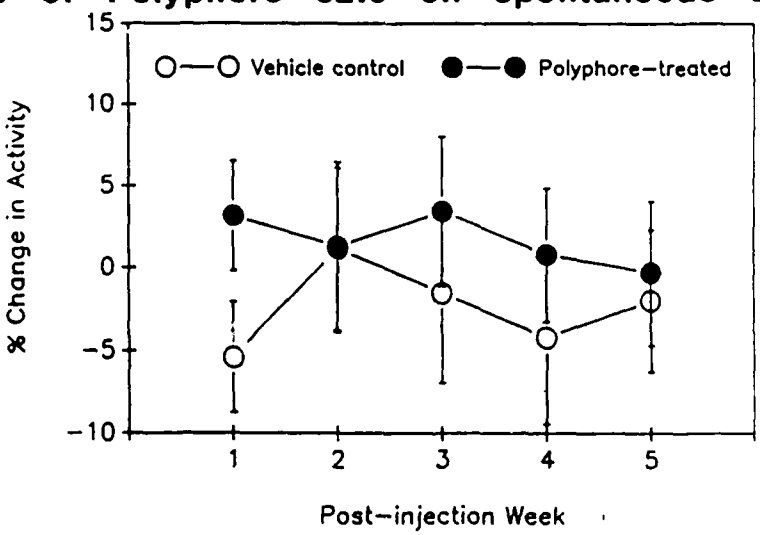



**Figure 6.** Nineteen rats were injected subcutaneously in the dorsum of the neck with 5 mg Polyphore 32:5 and 20 rats were injected similarly with the oil vehicle. Shown is the mean percent change in activity relative to values in the week prior to injections. Vehicle control rats tended to show a decrease in activity while copolymer-treated rats increased or showed less of a decrease

In all the spontaneous activity measurements the within-group variability was too high to allow any significant between-group differences. These data are being further analyzed for heterogeneity of variability and activity performance differences within individual sessions in the device.

Previous research showed that the copolymer attenuation of thymic involution in mice decreased three weeks after the injections. The rats in the previous studies were evaluated for four to five weeks after the injections, and it was hypothesized that the transient copolymer effect had disappeared by the time the animals were killed. In the next experiment animals were housed for three weeks after the injections, then killed. No other experimental manipulations were employed. No significant differences were found in body weight change, heart, spleen or thymus sizes, or in blood hematocrits.

In the next experiments we focused our attention on the water intake and urine output of rats in the controlled metabolic-cage environment with what we had determined appeared to be the optimal combination of animal strain, temperature and drug dosage. Two experiments were conducted with 24 Sprague-Dawley rats. In the first 12 rats were acclimatized to the colony, and baseline measurements of body weight, water intake and urine volume were taken for two weeks. Throughout the experiment urine specimens were frozen for later analysis. To determine if there was a differential effect in renal functioning between drug and control groups, animals were deprived of water on the 18th and 28th days after injections. Animals were killed for tissue samples five weeks after injections. There were no differential effects of water deprivation. Copolymer rats had a tendency toward lower urine osmolalities and higher urine volumes than vehicle rats.

In the other metabolic experiment, four of the 12 rats received weekly injections of the cold-solubilized copolymer that utilized no vehicle. There was no water deprivation in this experiment. Animals were killed 23 days post-injection. In this experiment no differences in urine volumes or osmolalities were found. Urine specimens from both of these experiments are still being analyzed for creatinine, urea and protein levels.

## Effects of Polyphore 32:5 on the change in body weight in rats

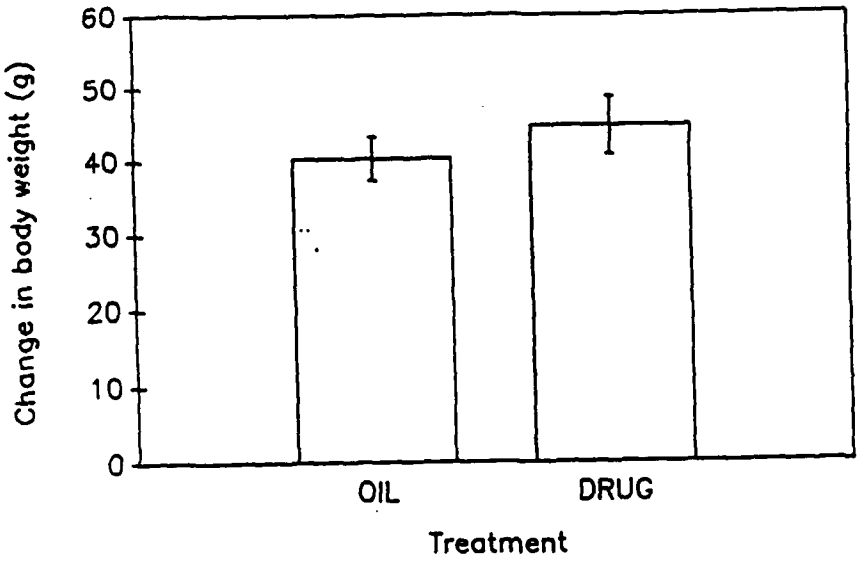



**Figure 7.** Rats were injected with 5 mg Polyphore 32:5 (n=29) or with the oil vehicle (n=30). The change in body weight is the rat's final body weight less the pre-injection body weight. Drug rats showed a greater increase in body weight.

## Effects of Polyphore 32:5 on thymus, spleen and heart weights as a proportion of body weight

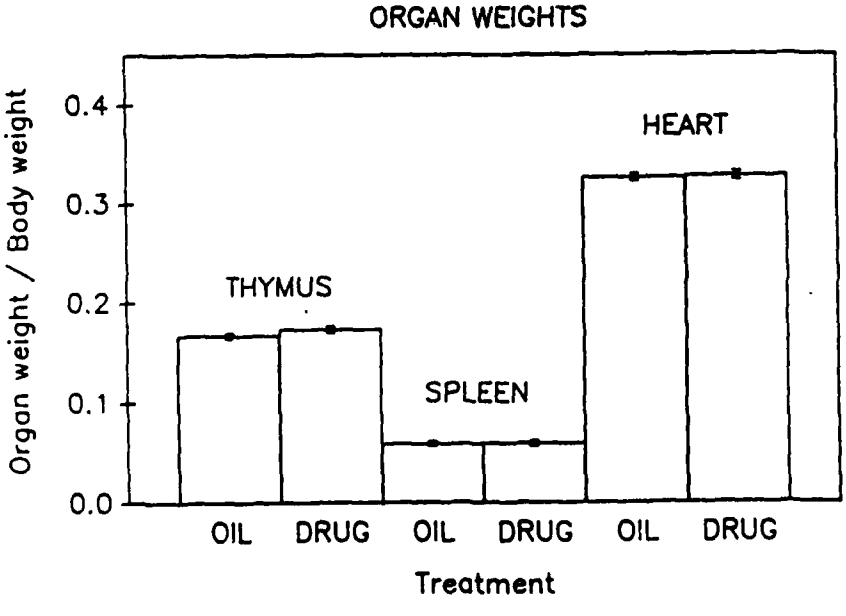



**Figure 8.** Rats were injected with 5 mg Polyphore 32:5 (n=29) or with the oil vehicle (n=30). Shown are the organ weights as a proportion of the final total body weight. Copolymer-injected rats tended to have larger organs.

Measurements of body weight change and thymus, spleen and heart weights as a proportion of body weights from comparable rat groups in the different studies were pooled. Copolymer animals tended to have a greater increase in body weights (initial body weight compared to final body weight), slightly larger thymuses and very slightly larger spleens and hearts (Figures 7 and 8). The differences were small and not significant.

One possible explanation for the consistent, but not significant, results of these experiments is that the copolymer may work only in some of the drug injected rats, but not in all. To test for this correlations are being conducted--e.g., is there a positive correlation between thymus size and activity? Correlation tests between several variable pairs have been completed and thus far no significant correlations have been found. Tests are not complete yet, but there is no indication that the copolymer works only in some of the rats.

Behavioral Studies in Stressed Rats

Food Deprivation Stress

In the first of these studies, nine of the rats were deprived of food for 5 days and then fed normally for another 5 days (until normal body weights were attained) prior to the determination of baseline measurements. Both food-deprived groups had higher baseline activity scores than the non-deprived groups, tended to greater decreases in activity post-injection, and showed greater variability than the non-deprived animals (Figure 9). The Polyphore-treated deprived animals showed these effects slightly more than the vehicle control animals. Again, none of the differences were statistically significant. Rats who had a prior period of food deprivation had a greater change in body weight than non-deprived rats and copolymer-treated deprived rats had a greater change than vehicle-control deprived rats. Food-deprived and non-food deprived animals did not differ in thymus, spleen and heart weights. Copolymer-treated deprived rats had smaller thymuses and hearts than vehicle-control deprived rats. None of these differences were significant.

## Effects of Polyphore 32:5 on the spontaneous activity of previously food-deprived rats

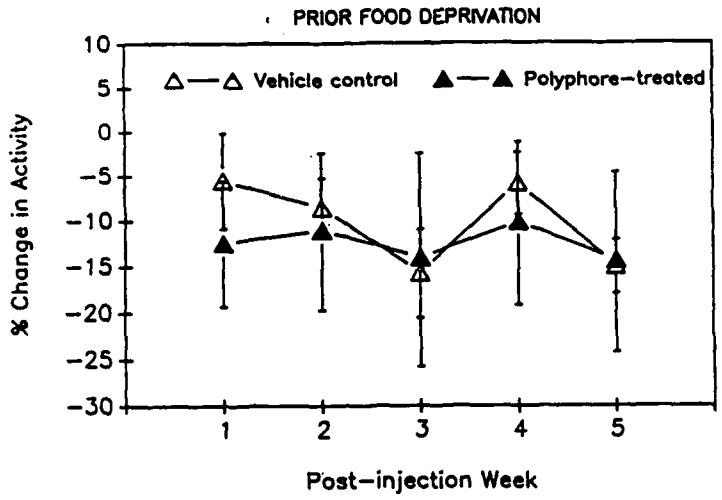



**Figure 9.** Rats were deprived of food for 5 days then injected two weeks later with copolymer (n=5) or oil vehicle (n=4). The y-axis is described in Figure 1. These rats showed a greater decrease in activity than the non-deprived rats shown in Figure 1, and the copolymer enhanced this decrease.

### Restraint Stress

In in vitro tests, Polyphore 32:5 has some properties similar to those of Substance P. It was recently shown that Substance P can protect the thymus from involution which results from exposure to a stressful situation. This experiment was designed to determine if Polyphore 32:5 would also protect rats from the thymic involution produced by restraint stress.

Initially, this experiment was carried out using one hour per day of restraint stress for four days. Because no thymic involution occurred, the restraint time was increased to 2 hrs daily in all subsequent experiments.

The experiment was run 3 times, in groups of 20 rats each. These groups are groups 15, 16, and 17. Male Lewis rats, age 90-94 days at the start of the experiment, were injected IP with Polyphore 32:5 in oil or just oil. Beginning the day after injection, half of these rats were subjected to two hours of restraint stress in Plexiglas restrainers daily for four consecutive days. On the fourth day rats in groups 16 and 17 only were also administered a 10 min activity test in a Digiscan device conducted immediately after restraint. All rats were sacrificed after restraint or restraint with activity test on the fourth day. Their hearts, spleens, and thymuses were removed and weighed for later analysis.

The results indicate that restraint stress did provoke thymic involution (see Fig. 10). Polyphore 32:5 did not protect from this effect. treated rats did have larger spleens than vehicle controls (Fig. 11); however, this effect was only significant for nonstressed rats. In the activity test, the stressed rats were slightly more active than the non-stressed, but this was only a trend.

**Effects of Polyphore 32:5 on thymus and spleen weights in stressed and non-stressed rats.**

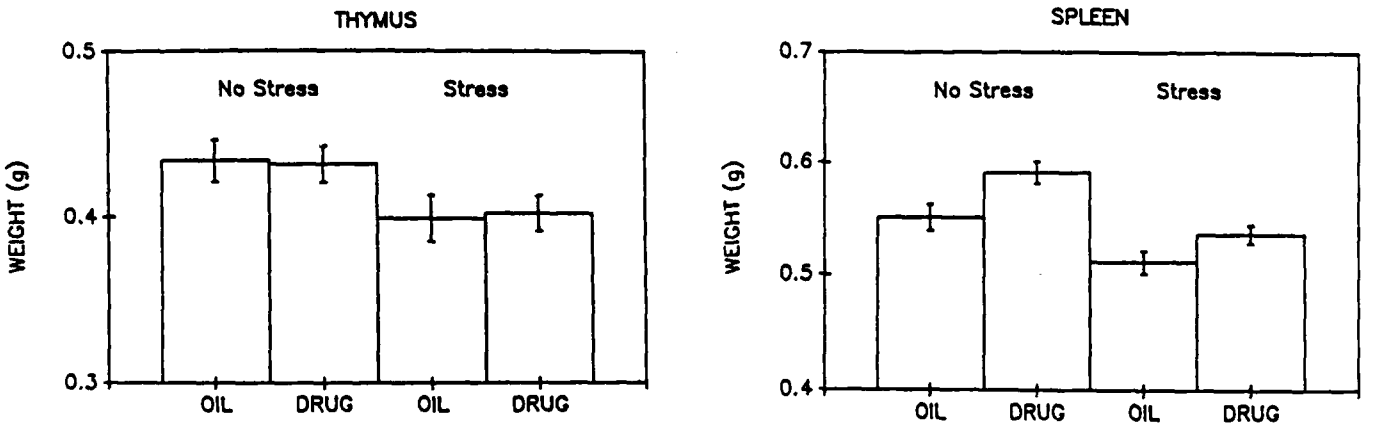



**Figure 10 and 11.** Male Lewis rats were injected with the copolymer or oil vehicle, then placed in restraining devices for 2 hours for 4 successive days (Stress Group) or allowed to remain in their home cages (No Stress Group). There were 15 rats in each of the 4 groups. The non-stressed rats had larger thymuses and spleens than the stressed rats. Copolymer-treated rats had larger spleens than oil vehicle rats.

Rewarding Brain Stimulation As a Stressor

A large literature exists describing deleterious effects of stress. Some data exist indicating that intracranial self-stimulation (ICSS), where animals respond to electrically stimulate certain brain sites, evokes large increases in circulating ACTH, corticosteroids, and beta-endorphins. Thus we have a condition which may be stressful, but which the animal avidly prefers. We have conducted some preliminary work on ICSS as a stressor.

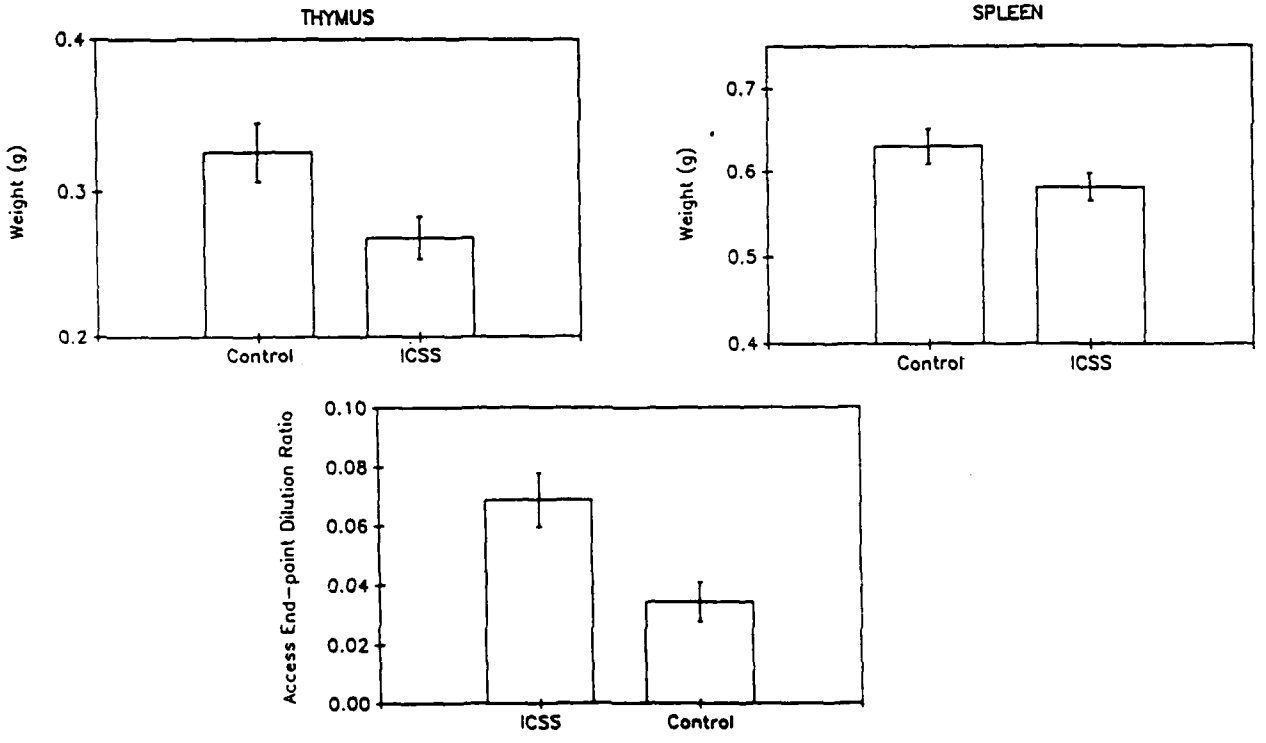
In these experiments, male Lewis rats were implanted with bipolar stainless steel electrodes aimed at the medial forebrain bundle in the lateral hypothalamic area. They were housed individually with free access to food and water in a room on a 12 hr lighting cycle.

One week after surgery, all animals were screened to determine if they would press a lever to electrically (150 msec trains of 0.5 msec 100 Hz

biphasic pulses) stimulate their brains. Eight of the most responsive animals were then allowed to self-stimulate daily for 5 weeks. Current was adjusted so that they responded between 1500 and 2500 times per session. The remaining 8 animals sat undisturbed in their cages. On day 19, all rats were injected I.P. with 0.5 ml of 5% sheep red blood cells (SRBC) by volume suspended in isotonic saline. On day 36, all rats were killed by $CO_2$ asphyxiation.

The results showed that the self-stimulating rats had significantly smaller thymuses (Figure. 12), a tendency toward smaller spleens (Figure.13 and a significantly smaller antibody titer (Figure 14) for SRBCs. Rats apparently like (to the point of thousands of bar-presses per hour) hypothalamic stimulation which may be immunologically detrimental. This pattern is reminiscent of that seen with drug abuse, where people compulsively self-administer chemicals which are often immunologically detrimental.

**Effects of 5 weeks ICSS on thymus and spleen weights and antibody formation for sheep red blood cells**




**Figures 12-14:** Rats which had self-stimulated for 5 weeks had significantly smaller thymuses and a trend toward smaller spleens. Their antibodies for sheep red blood cells were also lower as judged by the higher minimum concentration of plasma necessary to evoke sheep red blood cell agglutination.

Besides being intellectually interesting in being a new and uninvestigated form of stress, self-stimulation stress may be useful in confronting the problem that many other stress procedures are ethically troubling. Rather than inescapable shock, restraint, or other situations where the experimenter does something to the rat, with ICSS the rat does it to itself.

Our first ICSS experiment used weeks of stimulation. We have found that some of these immunological effects can be produced by one week of ICSS. In a recent experiment, 27 rats were divided into three groups: ICSS, electrode implanted but no ICSS controls, and unoperated controls. One week after surgery the ICSS group was allowed 7 days of self-stimulation. The results were that these animals had significantly smaller thymuses (see Figure 15) and nonsignificantly smaller spleens. SRBC antibody data are not available at this writing.

**Effects of 1 week ICSS on thymus weight**

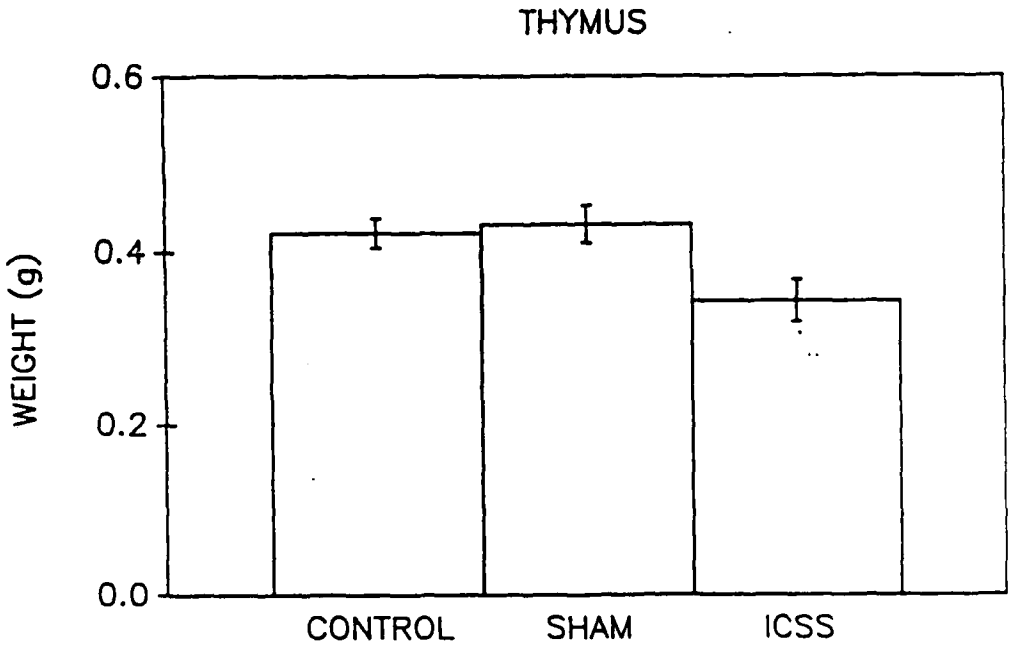



**Figure 15:** Rats self-stimulating as little as one week had significantly smaller thymuses.

## Specific Aims for Year 3

1. We have spent a great deal of time and effort on studies relating behavior to thymic size as a followup to our initial observations in this area. We have worked through enough variables to be convinced that the phenomenon is real but the variability in the system is so great as to preclude decisive dissection. Consequently, during the final year of this project, we will concentrate on leads suggested by earlier portions of this study which are likely to provide definitive answers to important questions. In particular, we will investigate the relationship between specific brain structures known to be involved in mood and motivation and the immune system. We will conduct more ICSS investigations, examining non-hypothalamic sites. We will also continue some preliminary work on brain catacholamine systems and the immune system.

2. Complete studies of the effects of copolymer on pituitary and extra-pituitary ACTH secretion. Studies in vivo will involve dexamethasone suppression, an effort to evaluate the ability of the copolymer to induce extra-pituitary ACTH. In the cell culture model, studies with the copolymers will be carried out in parallel with phorbol esters and various metabolic inhibitors in an effort to more clearly define the mechanisms involved. The model for these studies will be investigations on HL60 cells which were recently completed by a student in this department for the Ph.D. thesis.

3. Investigation of hematopoietic effects of the copolymers. Splenomegaly has been consistently observed in animals injected with the copolymers. Histologic sections have suggested that this is primarily hematopoietic with both red and white blood cells present. We will characterize the time course and nature of this effect on the hematopoietic system. This work will be done in collaboration with Dr. Elliot Winton who has established procedures for assaying various types of stem cells in culture.